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Using NMR to study fast dynamics in proteins: methods and applications

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Abstract

Proteins exist not as singular structures with precise coordinates, but rather, as fluctuating bodies that move rapidly through an enormous number of conformational substates. These dynamics have important implications for understanding protein function and for structure-based drug design. NMR spectroscopy is particularly well-suited to characterize dynamics of proteins and other molecules in solution at atomic resolution. Here, NMR relaxation methods for characterizing thermal motions on the picosecond-nanosecond (ps-ns) timescale are reviewed. Motion on this timescale can be conveniently captured by the Lipari-Szabo order parameter, S^2 , a bond-specific measure of restriction of motion. Approaches for determining order parameters are discussed, as are recent examples from the literature that link ps-ns dynamics with conformational entropy, allostery, and protein function in general.

Introduction

As part of the drug discovery repertoire, high-resolution protein structures from x-ray crystallography and NMR spectroscopy have been invaluable for structure-based drug design. While this will continue to be the case for the foreseeable future, it is also clear that single, static models, derived from crystals bathed in cryoprotecting cosolute at 100 K cannot reveal all the driving forces involved in binding. Advances in drug discovery techniques depend on an improved ability to quantify “invisible forces” such as the roles of solvent, ions, and protein dynamics in the overall binding free energy change[1]*. In considering the last of these invisible forces – protein dynamics – it has been known for some time that proteins are not static entities. Rather, they are ensembles of structures, and dynamic inter-conversion between these states is important for function. NMR relaxation is one of the methods of choice for studying protein dynamics, which can, along with additional NMR experiments, interrogate processes that span timescales of picoseconds to days. While slower dynamics is clearly of interest (reviewed elsewhere[2,3]), we focus here on NMR techniques for measuring fast (picosecond-nanosecond) protein dynamics due to

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their universality (these thermal motions are present in all proteins above the glass transition temperature), connection to conformational entropy, and implications for general function. This review aims to give the non-expert in NMR spectroscopy an introduction to the practical aspects of NMR relaxation methods for characterizing fast backbone and side-chain dynamics. Those dynamics are commonly interpreted as a single entity known as the order parameter, S^2 (not to be confused with entropy). In addition, we provide specific examples of how these tools have yielded valuable insights into biomolecular function. For more in-depth guides to both theory and implementation of backbone and side-chain relaxation experiments, readers are referred to thorough reviews by Stone[4]** and Wand[5]** respectively.

Link between protein dynamics and NMR relaxation

NMR signals (i.e. 1D, 2D, or n D “peaks”) are generated by perturbation (through application of radiofrequency pulses) of nuclear spins away from their equilibrium states. The process by which spins return to equilibrium is termed *relaxation*. The energy of NMR transitions dictates that relaxation does not occur spontaneously; rather, local fluctuations of the magnetic field at specific frequencies is required [6]. Thus, there is a link between molecular dynamics and relaxation in that local magnetic field fluctuations are caused by molecular motions. Because NMR transitions are quantized, the frequencies that cause relaxation are discrete and correspond to the resonance frequencies of the nuclei involved in the transitions. For typical biomolecular nuclei (^{15}N , ^{13}C , ^2H , and ^1H) in a high field spectrometer (e.g. 600 MHz ^1H resonance frequency), the relaxation-promoting fluctuations occur on the ps-ns timescale.

NMR relaxation methods for characterizing ps-ns dynamics of the protein backbone

The ^1H - ^{15}N peptide amide bond vector is ideally suited for NMR relaxation studies. First, as this bond vector moves with respect to the static magnetic field due to molecular tumbling or internal motions, the local field fluctuations sensed by the ^{15}N nucleus are dominated by the covalently attached ^1H dipole. Relaxation properties are therefore greatly simplified by the fact that there are no other NMR active nuclei attached. Second, relaxation measurements can be performed with variations on the simple and highly sensitive ^1H - ^{15}N HSQC experiment. Finally, the backbone amide bond represents a probe into the flexibility of every non-proline residue in proteins.

Molecular tumbling plays a dominant role in NMR relaxation. This is why molecular size is the main influence on NMR line shapes and experimental strategies. In order to gain valuable information about the internal dynamics of proteins, these effects must somehow be de-convoluted from the effects of tumbling. Nearly thirty years ago, Lipari and Szabo proposed a method for separating local, internal motions from tumbling[7,8]. Today, this “model-free” formalism is the dominant method for interpreting dynamics data reporting on processes faster than global tumbling (~ 10 ns for a 15 kD protein). To characterize ps-ns dynamics from ^{15}N relaxation, the ^{15}N R_1 and R_2 rates ($1/T_1$ and $1/T_2$, respectively) in addition to the $\{^1\text{H}\}$ - ^{15}N heteronuclear NOE are measured[9] (Figure 1). Experiments are ideally carried out at two static magnetic field strengths. This increases the robustness of fits and allows for fitting the data to more complicated models of motion (see below). These six relaxation parameters (R_1 , R_2 , and NOE at two fields) are then fit to model-free equations. ModelFree[10] and TENSOR2[11] are two free and downloadable fitting software packages with excellent documentation. In its basic form, there are three model-free parameters: τ_m , S^2 , and τ_e . Overall tumbling of the protein is described by τ_m . Once τ_m is initially extracted from the data, it is essentially fixed and bond-vector-specific (N-H in this case) values of S^2

and τ_e that correspond to motions within the molecular frame can be fitted to the relaxation data. The S^2 “order parameter” can vary from 0-1, with 1 corresponding to a rigid bond-vector and 0 corresponding to complete flexibility. The τ_e parameter is the characteristic time of the bond-vector motion, and typically takes on values of 0 to 100 ps but also up to a few nanoseconds in loops, termini, and some side chains. Thus, ^{15}N relaxation provides information on both the amplitude ($1-S^2$) and timescale (τ_e) for bond motion. Relaxation data for some residues fit better to model-free equations with different parameters besides the standard S^2 and τ_e pair. In total, there are five model-free models [10], and statistical criteria [12] built into most fitting packages aid the user in choosing which model best suits the data for each residue. For some residues, elevated R_2 values dictate that data should be fit to models containing a R_{ex} term which indicates motion on the μs - ms timescale. It is important to note that the pulse sequence for measuring R_2 rates contains an element designed to suppress the effects of exchange on the ms time scale. Thus the absence of elevated R_2 rates does not necessarily rule out chemical exchange. In addition, experimenters should use caution in interpreting elevated R_2 values, as anisotropic tumbling can cause the same effect in the *absence* of chemical exchange. This lesson was exemplified with the case of MDM2, a p53 binding protein with an N-terminal tail that is invisible in crystal structures. This tail was originally proposed to undergo chemical exchange based on an isotropic model of tumbling, but correct treatment of motional anisotropy changed the interpretation [13]*. Most model-free fitting packages guide the user through selecting the correct tumbling model. If the goal is to immediately discriminate between exchange and anisotropy, the R_1R_2 product is very sensitive to the former, but not the latter [14].

NMR relaxation methods for characterizing ps-ns dynamics of side chain methyl groups

Measuring dynamics at side-chain methyl groups is attractive because they are distributed throughout proteins and enriched within protein cores and binding interfaces. In addition, while backbone order parameters largely track with secondary structure (secondary structural elements are rigid, loops are flexible), the dynamic range of methyl order parameters is larger than the backbone counterparts (Figure 2) and are less predictable based on structure [5,15]. Values of methyl order parameters of 0.7 or less suggest the presence of rotameric switching in χ_1 or χ_2 [16,17]. For methyl groups, order parameters are typically obtained from ^2H spin relaxation. Briefly, proteins are expressed in media supplemented with ^{13}C -glucose, and ~50% D_2O such that methyl groups are a mixture of isotopomers (i.e. $^{13}\text{CH}_2\text{D}$, and $^{13}\text{CHD}_2$, where D is deuterium). Pulse sequences [18,19] select for signals originating from $^{13}\text{CH}_2\text{D}$ isotopomers and the degree of ^2H relaxation is then encoded in the intensity of ^1H - ^{13}C HSQC resonances (Figure 1). Typically, two experiments are performed, one measuring ^2H R_1 and the other, ^2H $R_{1\rho}$ (similar to R_2 but with no contribution from chemical exchange). It is important to note that some versions of the pulse sequences measure three spin coherences, $I_zC_zD_z$ and $I_zC_zD_y$ (where $I = ^1\text{H}$, $C = ^{13}\text{C}$, and $D = ^2\text{H}$). Thus, in order to obtain pure ^2H relaxation rates, a third experiment, I_zC_z , must be run and subtracted from the other two. These data are then fit to the model-free formalism. The order parameters correspond to C-CH₃ bond vectors and are referred to as S^2_{axis} , where ‘axis’ refers to the 3-fold symmetry axis collinear with the C-CH₃ bond and $S^2_{\text{axis}} = S^2_{\text{CH}_3}/0.111$. The sensitivity of these experiments degrades rapidly as the molecular weight of the protein increases beyond 30 kDa. Recent developments in assignment strategies, isotopic labeling schemes, and pulse sequence design have increased this size limit to over 100 kDa for some favorable cases [20]*.

Nature uses ps-ns fluctuations to modulate protein function

There is growing evidence that all proteins employ dynamics for function. While μ s-ms motions are often emphasized for being commensurate with the timescales of binding and catalysis, ps-ns motions are universal (not all proteins exhibit μ s-ms motions) and faster fluctuations have been proposed to facilitate exchange events on slower timescales[21]. The following is a sampling of cases where the linkage between dynamics on the ps-ns time scale and function is especially dramatic. Prior to the advent of NMR relaxation methods, protein flexibility was often inferred from x-ray crystal structures. For example, the structure of free calmodulin showed that the two lobes which must come together to envelope target peptides are separated by a 26-residue α -helix. In one of the first implementations of the model-free formalism, Bax and coworkers were able to reconcile this paradox and show that the middle of central “helix” is flexible in solution on the timescale of hundreds of picoseconds[22]. This central kink in the helix allows the two lobes to come together and bind targets. As another example, crystal structures of the free HIV protease implied the necessity for flexibility because the active sites were occluded by β -hairpins. Indeed, order parameters revealed that these β -hairpin “flaps” are flexible on the ps-ns timescale[23], with the fluctuations functioning to allow substrate access to and release of products from the active site. It was also shown that a high degree of ps-ns flexibility is advantageous to DNA binding proteins[24,25] as they must locate their target sites by navigating a vast array of non specific sites while sliding along the double helix. Lastly, fast fluctuations can modulate specificity. This is best exemplified by non-NMR studies of antibodies[26,27] showing that ps-ns flexibility is important for the broad specificity early on in affinity maturation and a stepwise decrease in flexibility of the binding site accompanies the stepwise increase in affinity for the target. NMR studies showed dynamics on this timescale influence specificity in a number of other systems[28,29].

Order parameters as a proxy for conformational entropy: Calmodulin as a model system

Structure-based drug design is employed because the relative binding affinities of a series of lead compounds can often be predicted based on the coordinates of static structural models. One of the ways NMR dynamics measurements can be of aid to the drug design community is if order parameters represent a window into molecular driving forces that are invisible to traditional structure based tools. Indeed, it was demonstrated almost two decades ago, that atomic coordinate fluctuation as measured by S^2 , are related to the canonical partition function, and hence the Gibbs free energy[30]. Soon thereafter, analytical expressions equating NMR order parameters to absolute conformational entropy were derived[31,32]. While the absolute entropies were highly dependent on the models used to describe the motion, relative entropies (i.e. between free and bound states) were robust and model independent. It was therefore predicted that *differences* in order parameters could be a proxy for differences in conformational entropy given the assumption that there are no correlated motions or the extent of correlated motions is the same in both states.

It was not until recently that Wand and coworkers performed the systematic study required to test this idea[33]**. Calorimetric measurements on calmodulin (CaM) binding to a series of six target peptides showed there is a large range in overall $T\Delta S$ for binding (~ 22 kcal mol⁻¹) despite the relatively constant overall ΔG (~ 3 kcal mol⁻¹)[34,35]. This large “dynamic range” in the overall entropy change, together with the fact that all six peptides are amphiphilic α -helices which bind to the same site on CaM made the system ideal. Side-chain order parameters were measured for free calmodulin (CaM) and CaM bound to the set of target peptides, and were subsequently used to calculate the conformational entropy changes for the binding reactions. The calculated entropies were compared to the total

calorimetric entropies, and a linear correlation between the two datasets was observed[33]. This provided the first experimental evidence that NMR order parameters are a proxy for entropy and are therefore reporters on a significant contributor to binding free energy.

While the linear relationship between the conformational entropy change derived from NMR order parameters ($\Delta S_{\text{conf}}^{\text{CaM}}$) and total entropy change (ΔS_{tot}) was highly suggestive, the quantitative contribution of $\Delta S_{\text{conf}}^{\text{CaM}}$ remained unknown due to the potential variability in the other factors influencing the total ΔS (equation 1)

$$\Delta S_{\text{tot}} = \Delta S_{\text{conf}}^{\text{CaM}} + \Delta S_{\text{conf}}^{\text{target}} + \Delta S_{\text{sol}} + \Delta S_{\text{RT}} \quad (1)$$

In equation 1: ΔS_{tot} represents the calorimetric entropy, the conformational entropy change is further divided into contributions from CaM and its target peptide, ΔS_{sol} represents changes in solvent entropy, and ΔS_{RT} represents entropy change due to loss of translational and rotational degrees of freedom upon binding. Based on this decomposition of the total entropy, Wand and coworkers reasoned that it would be possible to calibrate a “conformational entropy meter” if the changes in dynamics of the target peptide and changes in ΔS_{sol} are taken into account[36]*. In this study, Marlow *et al.* used the same series of six CaM-target pairs, measured the side-chain dynamics from the target peptide to access $\Delta S_{\text{conf}}^{\text{target}}$, and calculated ΔS_{sol} from crystal structures using accessible surface area (ASA) approaches. From this they observed the following linear relationship (equation 2):

$$(\Delta S_{\text{tot}} - \Delta S_{\text{sol}}) = m \left[\left(n_{\text{res}}^{\text{CaM}} \bullet \langle \Delta S_{\text{axis}}^2 \rangle^{\text{CaM}} + n_{\text{res}}^{\text{target}} \bullet \langle \Delta S_{\text{axis}}^2 \rangle^{\text{target}} \right) \right] + \Delta S_{\text{RT}} + \Delta S_{\text{other}} \quad (2)$$

(Note that order parameter “ S^2 ” is italicized to distinguish it from entropy. Marlow *et al.* substituted with “ O^2 ” for this reason.) Linearity of equation 2 demonstrates the following: 1) ΔS_{conf} is represented by the slope times the abscissa coordinate. 2) By scaling the average change in order parameter $\langle \Delta S_{\text{axis}}^2 \rangle$, by the number of residues in the calculation (n_{res} terms), binding partners with different numbers of NMR probes (assuming the ratio of probes used to total number of residues is relatively constant in the series) can be used to generate the model or as input for the “entropy meter”. 3) The y-intercept reports on $\Delta S_{\text{RT}} + \Delta S_{\text{other}}$, which is the same for each complex. 4) Variability in ΔS_{tot} can be completely accounted for by changes in methyl order parameters and ASA. This study showed that ΔS_{conf} and ΔS_{sol} were of roughly equal magnitude and opposite sign with both of these parameters being greater than the overall entropy change for binding. Therefore conformational entropy can be a true driving force in binding processes with the overall entropy change governed by the balance of the large and opposing solvation and conformational terms. It remains an open question as to whether this type of predictive power can be gleaned from similar studies on other homologous series and how related the binding reactions within a series must be for a linear relationship to be observed. We restate the caveat that accuracy of order parameter based methods for calculating differences in entropies requires that coupled motions are either absent or similar in both states. While success of the entropy meter approach shows this to be a reasonable assumption for calmodulin, recent molecular dynamics based calculations of absolute entropies in biomolecules show the effects of coupled motions can be large[37,38].

Finally, an alternative method for translating order parameters into conformational entropy draws upon molecular dynamics (MD) simulations. Because both order parameters and conformational entropy can be directly computed from MD simulations, Li and

Brüschweiler developed empirical simple scaling between the two for different classes of bond vectors[39]. The correspondence between S^2 and conformational entropy was remarkably linear. In principle, this allows for simple conversion of experimental S^2 values into conformational entropies.

ps-ns dynamics in the form of entropy can drive allostery

Targeting multiple proteins with different drugs has proven an effective strategy for battling resistance in HIV. An alternative is to target multiple sites on the same protein. Recently, it was shown that a pair of drugs, one binding to the ATP site, and the other binding to an allosteric site on Bcr-Abl is effective in overcoming drug resistant forms of chronic myeloid leukemia[40,41]. The promise of other drug targets with multiple, communicating sites provides the motivation to more deeply understand the rules governing allostery. Traditional models of allostery rely on conformational change to explain the phenomenon. However, it was proposed nearly three decades ago that it should be possible for allostery to be manifested by purely entropic means, that is, without net conformational change[42]. In this model, perturbation (i.e. mutation or binding) at one site affects the free energy change for a downstream event by influencing macromolecular fluctuations. Recently, NMR spin relaxation studies have revealed that entropically (dynamically) driven allostery is a bona fide mechanism utilized by nature. One example involves the catabolite activator protein (CAP), a dimeric transcription factor that binds two cAMP molecules with strong negative cooperativity. Popovych et al. used NMR probes of structure and dynamics to understand cooperativity in the N-terminal, cAMP binding domain (CBD) of CAP. Interestingly, binding of the first cAMP did not cause structural changes in the second binding site[43]*, leaving the possibility that dynamics are responsible for allostery. Backbone order parameters were then measured from apo-CBD₂, cAMP-CBD₂, and cAMP₂-CBD₂. These showed that binding of the first cAMP molecule caused very little change in the ps-ns dynamics of either subunit, while binding of the second molecule induced widespread quenching of dynamics (and loss of conformational entropy) in both subunits. The inferred conformational entropy mechanism of negative cooperativity was shown to be consistent with the overall entropies of binding measured by ITC. A second example involves a mutant in the cAMP binding domain of full length CAP. The S62F CAP variant binds to DNA with the exact same affinity as the wild-type, yet there is *27.5 kcal/mol difference in TΔS* between the two binding interactions[44]. Differences in backbone NMR order parameters between the cAMP₂-CAP₂ and cAMP₂-CAP₂-DNA states explained this observation in that the cAMP domain of the mutant was much more flexible than the wild-type in the DNA-bound state.

A final example of dynamically driven allostery comes from our lab studying the interaction between the third PDZ domain (PDZ3) from post-synaptic density 95 (PSD-95) and a C-terminal peptide from the CRIPT protein. PDZ3 has a C-terminal α -helix that is not present in most PDZ domains. Interestingly, this helix is phosphorylated at a location that may interfere with packing against the rest of the PDZ domain. To interrogate the role of a C-terminal helix in CRIPT binding we made a helical deletion mutant ($\Delta 7$ ct) and found $\Delta 7$ ct binds to the CRIPT peptide 20-fold more weakly than the wild-type despite the fact that the C-terminal helix does not contact the CRIPT peptide[45]*. The measured ΔH values for wild-type and $\Delta 7$ ct binding are exactly the same so the difference in binding is entirely entropic. Further, changes in NMR chemical shifts between free and bound deletion mutants were the same as those from the wild-type counterparts, effectively eliminating conformational change as a mechanism for the allosterically induced binding penalty. Instead, ²H relaxation studies show that truncation results in a global increase in the flexibility of methyl side chains in the unbound state such that the unfavorable *change* in conformational entropy upon peptide binding is increased relative to the wild-type.

Combining NMR relaxation and molecular dynamics tools overcomes the limitations of each

Biomolecules exist as ensembles of structures, and function is often carried out by lowly populated conformations that are not represented by crystal structures. For such cases, docking of rigid drug candidates to a rigid receptor will yield incorrect binding poses and misleading scores[46]. There are now many ways to incorporate flexibility into docking algorithms but serious limitations such as inadequate sampling of conformational space and bias towards the experimental structural model remain[47]. On the other side of the coin, the strength of NMR ps-ns relaxation methods lies in the fact that population weighted dynamics of the entire solution ensemble are extracted, yet order parameters do not give physical descriptions of the motions. Dobson, Vendruscolo, and colleagues recognized that the shortcomings of the simulation and NMR tools mentioned above could be eliminated if both approaches were combined. In the method termed dynamic ensemble refinement [48]* the CHARMM molecular mechanics force-field, NOE distances, and NMR order parameters (both backbone and side-chain) were used as restraints (equation 3) in generating an ensemble of ubiquitin structures.

$$E_{\text{tot}} = E_{\text{CHARMM}} + E_{\text{NOE}} + E_{S^2} \quad (3)$$

The ensemble was cross-validated against a large series of NMR residual dipolar coupling (RDC) measurements and was found to more faithfully represent these solution data than either the crystal structure or the NMR ensemble. This underscored the importance of the order parameters in defining the envelope of solution conformations. Further, because RDCs report on processes that occur on timescales from ns-ms, the ensemble encompasses conformational space that is inaccessible to short molecular dynamics simulations. This was a powerful demonstration of the complementary nature of modern NMR and computational tools.

Conclusions

Up until the first decade of the 21st century, with the exception of a minority of structural biologists, proteins were viewed as static structures. This view is undergoing a vast transformation throughout the biological sciences to one in which proteins are understood to dynamically sample a myriad of substructures within, or even outside, the overall fold. Our appreciation for this has likely been aided by the recognition that many functional proteins are intrinsically disordered. NMR spectroscopy has been instrumental in recording experimental evidence for protein dynamism. Protein motions occur on timescales from ps to days, and NMR methods are sensitive to essentially this entire timescale. Nevertheless, motions on the ps-ns timescale are particularly amenable to quantitative characterization of amplitudes of motion, through the “model-free” order parameter, S^2 . These “fast” order parameters are obtained from spin relaxation measurements (e.g., ^{15}N , ^{13}C , ^2H) and capture motion that may have a high degree of diffusive motion. Motions on slower timescales are not easily characterized by order parameters, yet those motions may be more concerted and switch-like. In terms of protein function and drug design, fast backbone and side-chain motions appear to have their largest influence through conformational entropy, as discussed in this review. However, correlating fast dynamics (i.e., S^2) with function in other ways is often not straightforward, even though vital connections likely exist. This difficulty may stem from the relatively simple information contained in S^2 . Future progress for correlating fast dynamics with function is likely to depend on combining NMR and MD simulations, as well as investigators’ ingenuity in experimental design to probe these relationships.

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Figure 1.

Basic flow-chart for characterization of ps-ns dynamics by NMR. A pictorial representation of T_2 relaxation is presented in the first quadrant. The presence of a static magnetic field (B_0) results in a bulk magnetization (grey arrow) aligned with B_0 . An RF pulse rotates the bulk magnetization into the xy plane. The individual spins de-phase during the relaxation time (T), leading to the exponential decrease in the bulk magnetization as a function of T . The rate of loss of spin coherence is referred to as the spin-spin relaxation rate (R_2). Molecular tumbling and internal fluctuations affect this rate of relaxation. Relaxation is monitored by acquisition of 2D HSQC type spectra with variable times of T (upper right quadrant). The spectra are ^1H - ^{15}N and ^1H - ^{13}C for ^{15}N and ^2H relaxation, respectively. ^{15}N relaxation typically involves measurement of two rates, R_1 and R_2 , and the $\{^1\text{H}\}$ - ^{15}N heteronuclear NOE; ^2H relaxation involves measurement of R_1 and $R_{1\rho}$. To obtain relaxation rates, the decays in resonance intensities are fit to single exponential equations (lower right quadrant). These raw relaxation parameters are subsequently fit to Lipari-Szabo model-free equations in order to obtain parameters describing amplitude (the order parameter, S^2), and characteristic time (τ_e) of bond vector motion (lower left quadrant). Note that the model-free parameters report on the amide ^1H - ^{15}N bond vector (black) and the C-CH₃ symmetry axis (alanine example in red) for ^{15}N and ^2H relaxation, respectively.

Figure 2.

Side-chain flexibility is more heterogeneously distributed than main-chain flexibility. Backbone amide S^2 values (black) and side-chain methyl S^2_{axis} values (red) for Ca^{2+} -bound calmodulin are plotted. Note that the backbone values are tightly clustered around 0.9 with the exceptions of loops and the central helix (α -helices and β -sheets are depicted by cylinders and arrows respectively at the top of the figure). The side-chain methyl order parameters are more heterogeneous and have no relation to the protein secondary structure. Data were obtained from the Biological Magnetic Resonance Data Bank (accession number 15188).